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ORIGINAL ARTICLE

Persistence of enterohaemorrhagic and nonpathogenic *E. coli* on spinach leaves and in rhizosphere soil*

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Abstract

Aims: Survival of *Escherichia coli* O157:H7 and nonpathogenic *E. coli* on spinach leaves and in organic soil while growing spinach in a growth chamber was investigated.
Methods and Results: Spinach plants were maintained in the growth chamber at 20°C (14 h) and 18°C (10 h) settings at 60% relative humidity. Five separate inocula, each containing one strain of *E. coli* O157:H7 and one nonpathogenic *E. coli* isolate were applied to individual 4-week-old spinach plants (cultivar 'Whale') grown in sandy soil. Leaf and soil inocula consisted of 100 μl, in 5 μl droplets, on the upper side of leaves resulting in 6·5 log CFU plant^{-1} and 1 ml in soil, resulting in 6·5 log CFU 200 g^{-1} soil per plant. Four replicates of each plant shoot and soil sample per inoculum were analysed on day 1 and every 7 days for 28 days for *E. coli* O157:H7 and nonpathogenic *E. coli* (by MPN) and for heterotrophic plate counts (HPC). *Escherichia coli* O157:H7 was not detected on plant shoots after 7 days but did survive in soil for up to 28 days. Nonpathogenic *E. coli* survived up to 14 days on shoots and was detected at low concentrations for up to 28 days. In contrast, there were no significant differences in HPC from days 0 to 28 on plants, except one treatment on day 7.
Conclusions: *Escherichia coli* O157:H7 persisted in soil for at least 28 days. *Escherichia coli* O157:H7 on spinach leaves survived for less than 14 days when co-inoculated with nonpathogenic *E. coli*. There was no correlation between HPC and *E. coli* O157:H7 or nonpathogenic *E. coli*.
Significance and Impact of the Study: The persistence of nonpathogenic *E. coli* isolates makes them possible candidates as surrogates for *E. coli* O157:H7 on spinach leaves in field trials.

Introduction

Escherichia coli O157:H7 have been involved in many disease outbreaks in the United States, with estimates of 110 000 *E. coli* O157:H7 infections, 3200 hospitalizations and 61 associated deaths occurring annually (CDC 2008). The number of foodborne illness associated with consumption of fresh produce has increased substantially during the past 15 years (Olsen *et al.* 2000). In 2006, an outbreak of *E. coli* O157:H7 was linked to consumption of fresh, bagged, baby spinach, with 26 states in the US and Canada reporting 205 cases of illness and three deaths (CDC 2006). Contaminated product was traced to a specific production date at a processing plant and to fields located on four ranches. Another *E. coli* O157:H7 outbreak associated with shredded lettuce resulted in 71 cases of illnesses, 53 hospitalizations and 8 cases of hemorrhagic uremic syndrome (HUS) (California Food Emergency Response Team 2008). In this outbreak, inadequate backflow protection devices might have contaminated the water used for irrigation of lettuce fields with dairy faeces. In both outbreaks, produce contamination was suspected to have occurred on the farm on which the produce was grown.

Contamination of produce may occur along the food production chain "from farm to fork". Sources of preharvest contamination include faeces, soil, irrigation water, improperly composted manure, air, wild and domestic animals, equipment and human handling (Beuchat 1996; Wachtel *et al.* 2002b). Pathogen transfer from manure to vegetables via contaminated soil is well established (Natvig *et al.* 2002; Islam *et al.* 2004a,b,c; Barak and Liang 2008). Irrigation water that directly contacts the harvestable commodity may also contribute to *E. coli* O157:H7 contamination of fresh produce at the primary production stage on farm (Ackers *et al.* 1998). The pathogen has also been shown to survive for at least 50 days in water collected from a range of surface sources including rivers, streams, municipal reservoirs and lakes (Wang and Doyle 1998; Avery *et al.* 2008). Mesclun mix (leafy greens mixture) implicated in a multistate outbreak in 1996 was contaminated by irrigation water (Hilborn *et al.* 1999). Previous studies have reported persistence of *E. coli* O157:H7 on lettuce, cabbage and other produce when irrigated with contaminated water (Solomon *et al.* 2002, 2003; Wachtel *et al.* 2002a,b; Islam *et al.* 2004c).

Persistence of *E. coli* O157:H7 in the field depends on numerous factors. Results of recent studies indicated that *E. coli* O157:H7 can survive in fields for 4–8 weeks (Ibekwe *et al.* 2004; Johannessen *et al.* 2005). However, *E. coli* O157:H7 persisted for 154–217 days in soils amended with manure compost inoculated with 10^7 CFU g^{-1} and was detected in lettuce for up to 77 days after seedlings were planted (Islam *et al.* 2004a). Similarly, *E. coli* O157:H7 was detected from carrots 168 days after application of this same inoculated manure compost (Islam *et al.* 2005). Although pathogen contamination of vegetables by irrigation water and composted manure has been studied, little information is available comparing the survival of *E. coli* O157:H7 with nonpathogenic *E. coli* on spinach leaves and in soil. Manure contamination of leafy greens may include the presence of *E. coli* O157:H7 and nonpathogenic *E. coli*. The objective of this study was to evaluate the persistence of *E. coli* O157:H7 and nonpathogenic *E. coli* on spinach plants and in organic soil when simultaneously present. The efficacy of using nonpathogenic *E. coli* as surrogate for *E. coli* O157:H7 was evaluated.

Materials and methods

Spinach plants

Spinach (*Spinacia oleracea* L.) cultivar 'Whale' (Meyer Seed Co., Baltimore, MD) was selected from a screening trial (results not shown) because of its vigorous growth in the growth chamber setting. The baby spinach 'Whale' cultivar is a very uniform dark green baby spinach with good heat tolerance and is frequently used in the mid-Atlantic region of the US. Untreated seeds were added at three per cone-tainer (8·25 cm × 1·5 cm; Stuewe & Sons, Inc., Corvallis, OR, USA) containing a new glass fibre wad at the base (to prevent soil from passing out of the drainage holes); and 200 g soil, sieved (2 mm) Keyport sandy loam (3% organic matter) collected from the USDA-BARC organic plots (USDA Beltsville Agricultural Research Center, Beltsville, MD, USA). Cone-tainers were placed upright into retaining slots of cone-tainer trays (RL98, Stuewe & Sons, Inc., Corvallis, OR, USA) which suspend the base of the cone-tainers above the level of drainage water to ensure that no cross contamination between cone-tainers occurs. After germination in the dark, plants were grown and maintained in the BSL-2 growth chamber at 20°C and 18°C, for day (14 h) and night (10 h), respectively, at 60% RH and 240 μmoles per m^2 s^{-1} light intensity. Plants were irrigated carefully to avoid splashing the deionized water and container soil onto leaves; water was supplied as needed to maintain soil moisture at field capacity and plant turgor. Fish emulsion (Organic Neptune's Harvest Fish Fertilizer, 2-4-1, Gloucester, MA, USA) prepared as 8 ml/l was applied at 50 ml per cone-tainer 10 days prior to inoculation.

Bacterial strains

Five separate inocula treatments, each containing one strain of *E. coli* O157:H7 and one nonpathogenic *E. coli* isolate (from plants or soil), were used in the study (Table 1). *E. coli* O157:H7 strains RM 4406 and RM 1918 (lettuce outbreak isolates) and RM 4407 (spinach outbreak isolate) were kindly provided by Robert Mandrell (US Department of Agriculture, Agricultural Research Service, Albany, CA, USA). Two nontoxigenic *E. coli* O157:H7 strains were also used: strain 3704 isolated from a farm drain was kindly provided by K. Killham (University of Aberdeen, Scotland), and strain B6914 was provided by Pina Fratamico (US Department of Agriculture, Agricultural Research Service, Wyndmore, PA, USA). Five nonpathogenic commensal strains of *E. coli* MW416, MW 423 and MW 425 (cabbage isolates), PM 3823 (Spinach isolate)

Table 1 Strains of *Escherichia coli* used in each of five inocula treatment combinations

Treatment Number	*E. coli* O157:H7 strain	Nonpathogenic *E. coli* strain
1	RM 4406	PM 3823
2	RM 1908	MW 425
3	RM 4407	MW 416
4	B6914	MW 423
5	3704	PM 3954
6	Uninoculated control	Uninoculated control

and PM 3954 (lettuce isolate) were used from our Environmental Microbial and Food Safety Laboratory culture collection. Frozen cultures of each strain were partially thawed at room temperature (*c.* 22°C) for 15 min and streaked onto tryptic soy agar slants (TSA; Becton Dickinson) and incubated at 37°C for 24 h.

Inoculum preparation

Strains (Table 1) from TSA slants were grown in TSB for 24 h at 37°C after which cells were pelleted by centrifugation (5000 ***g***, 15 min) and washed with phosphate-buffered saline (PBS) twice. The cell pellets were resuspended in PBS, and 50 μl of the suspension of each strain was transferred to an individual tube of bovine faecal solids extract (45 ml) and incubated at 37°C for 48 h. The bovine faecal solids extract was prepared 24 h prior as sterile deionized water extract (1 : 10 dilution) from centrifuged sterile dairy manure solids (obtained from the USDA-ARS Holstein dairy herd, Beltsville, MD, USA). Extracts were filter-sterilized (0·22 μm filter unit, Millipore, Billerica, MA, USA) and tested for sterility by plating slurry on TSA and incubating at 37°C for 24 h.

Escherichia coli inocula consisted of one *E. coli* O157:H7 strain and one nonpathogenic *E. coli* strain (Table 1), and these strains were combined in equal volumes of the sterile extracts. Leaves of 4-week-old spinach plants were inoculated with 100 μl (as 5 μl droplets) on *c.* 5 cm long spinach leaves to obtain *c.* 6·5 log CFU plant^{-1}. Soil was inoculated by injecting 1 ml of *E. coli* inoculum alongside the stem 1·0 cm below the soil surface. The inoculum concentration in soil was 6·5 log - CFU 200 g soil^{-1} per plant. Plants inoculated with manure extracts containing no inocula of *E. coli* were used as controls.

Bacterial analysis

Four replicates of each plant shoot and soil sample per treatment were analysed on day 1 and every 7 days for 28 days for *E. coli* O157:H7, nonpathogenic *E. coli* and heterotrophic plate counts (HPC). The HPC were also determined on the day of inoculation (day 0). Plant shoots (leaves and a stem, 2 g) were harvested aseptically by cutting the stem just above the soil surface with sterile scissors; shoots were transferred aseptically into sterile whirl-pak bags (Nasco, Ft. Atkinson, WI, USA) and kept at 4°C (for up to 20 min) before 10 ml of buffered peptone water (BPW; Becton Dickinson, Sparks, MD, USA) was added. New spinach plants were harvested at each sampling time for collecting plant shoots. Shoots in BPW were sonicated for 30 s (Astrason ultrasonic cleaner, Plainview, NY, USA), after which the suspension was pummelled for 2 min (Bagmixer, Interscience, St Nom, France). Suspensions were dispensed to mEHEC broth (Biocontrol systems, Inc., Bellevue, WA, USA) and serially diluted for an 8-tube MPN assay in 96-deepwell (2·2 ml cell^{-1}) microplates (Fisher Scientific, Newark, DE, USA) and incubated at 42°C for 18 h. After enrichment, each suspension was streaked on sorbitol-MacConkey media supplemented with 0·05 mg l^{-1}cefixime and 2·5 mg l^{-1} potassium tellurite (CT-SMAC, Becton Dickinson) and incubated at 40°C for 24 h. Randomly selected colonies (three per plate) were confirmed by latex agglutination assay (Remel Inc., Lenexa, KS, USA). Results were calculated and expressed as MPN shoot^{-1}. For nonpathogenic *E. coli* MPN, mFC broth (Becton Dickinson) was used in 8-tube MPN assay for enrichment at 37°C for 24 h followed by streaking of each suspension on MacConkey agar (Becton Dickinson). After incubation of 24 h at 44°C, positive colonies were counted, and the results were expressed as MPN per shoot. HPC were determined by plating serial dilutions onto tryptic soy agar (TSA) supplemented with 50 mg l^{-1} cyclohexamide (Sigma-Aldrich, St Louis, MO, USA) followed by incubation at 37°C for 24 h. Populations of *E. coli* O157:H7 and nonpathogenic *E. coli* within soil samples were assessed by plating on CT-SMAC and MacConkey agar, respectively, with incubation at 37°C for 24 h.

Statistical analysis

A randomized complete block design was used with 4 replicates per treatment. The bacterial populations obtained at each sampling period and with each treatment were converted to log CFU plant^{-1} and log CFU 200 g^{-1} soil for shoots and soil samples, respectively. The data obtained from four replicates were analysed by a two-way ANOVA using 'PROC MIXED' (SAS 8.2, Cary, NC, USA) for effects of the treatment and sampling period.

Results

Persistence of *E. coli* O157:H7

Escherichia coli O157:H7 persisted for less than 14 days on young spinach plant shoots when inoculated with droplets of aqueous manure extracts (Fig. 1), although differences in the persistence of individual *E. coli* O157:H7 were observed. Initial *E. coli* O157:H7 populations on plant shoots enumerated by MPN on day 1 were *c.* 2 log CFU shoot^{-1} for all strains except the strain B6914 (0·38 log). Only *E. coli* O157:H7 RM 1908 and 3704 persisted after 7 days exposure to spinach shoots. No *E. coli* O157:H7 strains were recovered from spinach shoots on 14, 21 and 28 days postinoculation. In soil, all

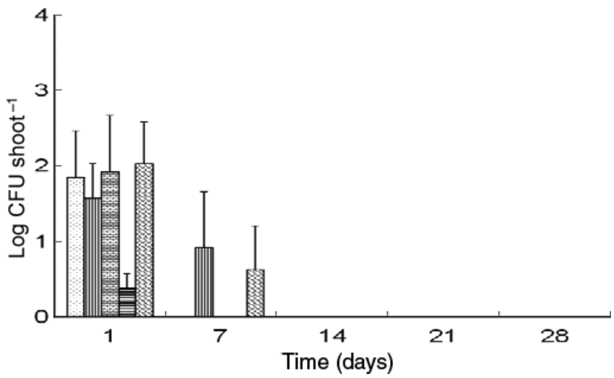

Figure 1 Survival of *Escherichia coli* O157:H7 on spinach leaves spot-inoculated (6·5 log CFU shoot^{-1}) onto 4-week-old spinach plants. *Escherichia coli* O157:H7 were co-inoculated with nonpathogenic *E. coli* strains. Results shown are mean values of four replicates. Error bars indicate the standard error. ▫, RM 4406; ▥, RM 1908; ▦, RM 4407; ▤, B6914; ▨, 3704.

E. coli O157:H7 strains with initial concentrations at *c.* 6·5 log CFU 200 g soil^{-1} declined over time but persisted through the 28-day study. Populations of *E. coli* O157:H7 strains in soil analysed by MPN on day 28 were as follows: RM 4406 (3·12 log) > strain 3704 (2·54 log) > B6914 (2·28 log) > RM 1908 (2·06 log) > RM 4407 (1·09 log).

Persistence of nonpathogenic *E. coli*

Initial *E. coli* MPN populations on spinach leaves varied with strain (Fig. 2). Recovery of nonpathogenic strain MW416 (0·46 log shoot^{-1}) on day 1 was significantly lower than the recovery of strains PM3823 (2·82 log shoot^{-1}), PM3954 (2·49 log shoot^{-1}) and MW425 (1·88 log shoot^{-1}) on day 1. Likewise, significantly lower populations of strain MW416 were recovered on day 7 in comparison with the populations of strains PM3954 and MW425. Nonpathogenic *E. coli* were recovered from spinach leaves for at least 14 days from all treatments. Two of the five nonpathogenic *E. coli* strains (MW 416, MW 425) persisted on plant shoots for up to 28 days. The effect of sampling period also influenced recovery of all nonpathogenic *E. coli* strains but strain MW 416. Persistence of these nonpathogenic *E. coli* strains on spinach leaves was similar ($P > 0{\cdot}05$) after 7 days of inoculation with the exception of strain PM3823, persistence of which was lower ($P < 0{\cdot}05$) compared to that on day 1. Populations of strains PM 3823, PM 3954, MW 423 and MW 425 detected on spinach leaves after 21 days were significantly lower than their corresponding populations recovered on day 1. Nevertheless, nonpathogenic *E. coli* populations of these strains recovered at 21 days were similar ($P > 0{\cdot}05$) to their corresponding populations on day 28. Overall, recovery of nonpathogenic *E. coli* from plant shoots varied widely, and there was no clear trend observed among the strains examined. Lack of recovery of nonpathogenic *E. coli* in soil samples was because of the number of non-*E. coli* bacteria present and the lack of selectivity of MacConkey agar for *E. coli* from soil. MPN assays of soil samples, carried out at 28 days because of difficulty in identification of *E. coli* by direct plating, detected 4·43–5·53 log CFU 200 g soil^{-1}.

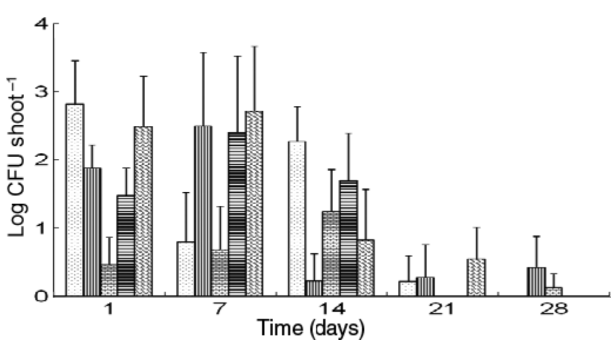

Figure 2 Survival of nonpathogenic *Escherichia coli* on spinach leaves spot-inoculated (6·5 log CFU shoot^{-1}) on 4-week-old spinach plant and grown in growth chamber. Nonpathogenic *E. coli* strains were co-inoculated with *E. coli* O157:H7 strains. Results shown are mean values of four replicates. Error bars indicate the standard error. ▫, PM 3823; ▥, MW 425; ▦, MW 416; ▤, MW 423; ▨, PM 3954.

Recovery of total heterotrophic bacterial counts (HPC)

Initial HPC on plant shoots were *c.* 6·5 log CFU shoot^{-1} for each of the five treatments containing *E. coli* inocula, and these populations were significantly higher than the HPC (5·10 log CFU shoot^{-1}) from the uninoculated control (treatment 6) applied to spinach plants (Table 2). The HPC increased gradually or remained stable in all treatments except treatment 5 where the counts decreased significantly by day 1. The HPC increased in four of the five treatments and in control plant shoots by day 21. The HPC populations obtained on day 21 in control and treatment 4 were significantly higher than the corresponding populations obtained for these treatments on day 0. Overall, the HPC declined in all treatments over the duration of the 28-day study. Furthermore, significantly lower HPC were observed on day 21 than on day 28 for treatments 3 and 4. The effect of treatment on specific sampling days was evident with two of the five treatments. The HPC obtained after 7 days with treatment no. 5 (6·25 log CFU shoot^{-1}) were significantly lower than the HPC populations with treatment no. 2 (7·06 log CFU shoot^{-1}) and control (7·05 log CFU shoot^{-1}) at 7 days. Likewise, HPC populations obtained with treatment no. 4

Table 2 Heterotrophic plate counts (HPC) on spinach leaves co-inoculated with *Escherichia coli* O157:H7 and nonpathogenic *E. coli* and grown in growth chamber

Treatment*	Bacterial populations (log CFU shoot^{-1})†,‡					
	Time (Days)					
	0	1	7	14	21	28
1	6·68 ± 0·35 a	6·28 ± 0·38ab	6·91 ± 0·57a	6·65 ± 0·63ab	6·37 ± 0·47ab	5·93 ± 0·77b
2	6·60 ± 0·17ab	6·40 ± 0·35ab	7·06 ± 0·56a	6·18 ± 0·53b	7·04 ± 1·03a	6·42 ± 0·50ab
3	6·64 ± 0·36ab	6·08 ± 0·50bc	6·51 ± 0·39ab	6·63 ± 0·30ab	7·12 ± 0·56a	5·70 ± 0·95c
4	6·26 ± 0·46ab	6·42 ± 0·64abc	6·53 ± 0·43bc	7·09 ± 0·32c	6·97 ± 0·11c	5·74 ± 0·64a
5	6·39 ± 0·35a	5·44 ± 0·86b	6·25 ± 0·65a	6·70 ± 0·26a	6·93 ± 0·30a	6·38 ± 0·69a
6	5·10 ± 0·94a	6·52 ± 0·54bcd	7·05 ± 0·32d	6·73 ± 0·37cd	6·26 ± 0·68bc	5·89 ± 0·60b

*Treatment consists of co-inoculation of *E. coli* O157:H7 and nonpathogenic *E. coli* strains (1:RM 4406 and PM 3823; 2: RM 1908 and MW 425; 3: RM 4407 and MW 416; 4: B6914 and MW 423; 5: 3704 and PM 3954; 6: Uninoculated control).
†Means in the same row with different letters (a,b,c) are significantly different.
‡SD, standard deviation.

on day 14 (7·09 log CFU shoot^{-1}) and day 21 (6·97 log CFU shoot^{-1}) were significantly higher than the corresponding HPC with treatment no. 2 (6·18 log CFU shoot^{-1}) on day 14 and with treatment no. 1 (6·37 log CFU shoot^{-1}) on day 21. Total bacterial populations in soil were *c.* 7 log CFU 200 g^{-1} (Table 3) which increased ($P < 0·05$) after 7 days in treatments 1, 4, and 5 and control. HPC in soils declined significantly after 14 days in all treatments with the exception of treatment 4, followed by marginal increase in HPC for all treatments at 28 days. HPC recovered in soil at 28 days were marginally higher than the initial HPC (day 0) of corresponding treatment.

Discussion

Microbial contamination of produce may occur anytime during the primary production, harvesting and processing/packing stages. Because there is no 'kill-step' during packing of raw produce, identifying source of preharvest contamination of fresh produce is necessary to control foodborne illness and to avoid potential produce recalls. Persistence of *E. coli* O157:H7 in the crop production environment has been studied in laboratory as well as under field conditions. *E. coli* O157:H7 persisted for 77 and 177 days on leaf lettuce and parsley, respectively, when grown in soils amended with *E. coli* O157:H7-contaminated compost and irrigated with contaminated water (Islam *et al.* 2004a). Further, they reported persistence of *E. coli* O157:H7 for 74 and 168 days on onions and carrots, respectively, when these produce were irrigated with contaminated water or manure compost (Islam *et al.* 2005). However, comparison of results from individual studies is quite difficult because of variations in type of produce and cultivars, methods of inoculation,

Table 3 Heterotrophic plate counts (HPC) in soil co-inoculated with *Escherichia coli* O157:H7 and nonpathogenic *E. coli* and grown in growth chamber

Treatment*	Bacterial populations (log CFU 200 g soil^{-1})†,‡					
	Time (Days)					
	0	1	7	14	21	28
1	6·91 ± 0·08a	7·16 ± 0·19a	7·79 ± 0·05b	7·91 ± 0·15b	6·98 ± 0·10a	7·26 ± 0·07a
2	6·96 ± 0·11a	6·92 ± 0·18a	7·33 ± 0·39ab	7·65 ± 0·25b	6·95 ± 0·17a	7·22 ± 0·12ab
3	6·83 ± 0·10a	6·88 ± 0·13a	6·87 ± 1·27a	7·71 ± 0·14b	6·98 ± 0·15a	7·31 ± 0·38a
4	6·89 ± 0·18ab	6·69 ± 0·14b	7·55 ± 0·70d	7·47 ± 0·07cd	7·08 ± 0·08abc	7·38 ± 0·30bcd
5	6·94 ± 0·22a	6·99 ± 0·21a	7·91 ± 0·91b	7·79 ± 0·18b	7·00 ± 0·07a	7·12 ± 0·18a
6	6·95 ± 0·13a	7·11 ± 0·22a	7·79 ± 0·19c	7·64 ± 0·14bc	6·76 ± 0·09a	7·25 ± 0·07ab

*Treatment consists of co-inoculation of *E. coli* O157:H7 and nonpathogenic *E. coli* strains (1:RM 4406 and PM 3823; 2: RM 1908 and MW 425; 3: RM 4407 and MW 416; 4: B6914 and MW 423; 5: 3704 and PM 3954; 6: Uninoculated control).
†Means in the same row with different letters (a,b,c) are significantly different.
‡SD, standard deviation.

bacterial strains and methods of analysis. These studies did not directly apply bacterial inocula to foliar surfaces. In our study, *E. coli* O157:H7 survived for <14 days when directly inoculated onto spinach leaves. It is possible that spinach foliar surfaces may be less conducive to support *E. coli* O157:H7 survival than other leafy greens. The presence of nonpathogenic *E. coli* could have also attributed to the shorter persistence in the present study. The growth temperature could have influenced *E. coli* O157:H7 persistence in our study. Mitra *et al.* (2009) observed persistence of *E. coli* O157:H7 on spinach leaves after 14 days when grown at 24°C. They also demonstrated the effect of spinach cultivar on growth and persistence of *E. coli* O157:H7. The smooth leaves of 'Whale' cultivar might have resulted in weak *E. coli* O157:H7 attachment to spinach leaves. *Escherichia coli* O157:H7 survived on lettuce leaves for up to 30 days following spray irrigation (Solomon *et al.* 2003). However, in this study, plants were spray-irrigated multiple times with contaminated water. In our work, we were unable to recover *E. coli* O157:H7 14 days postinoculation after the single inoculation event on day 0.

Survival of *E. coli* O157:H7 in soil depends on multiple factors including type of produce, soil type and its nutrient and biological content and activity (competition, antibiosis, predation, parasitism), fertilization, irrigation, cropping and soil management practices, and climatic effects (Natvig *et al.* 2002; Ibekwe *et al.* 2004). *Escherichia coli* O157:H7 and *Salmonella* survived in the soil for up to 1 month after application of contaminated manure to both the sandy and clay soils (Nicholson *et al.* 2005). *Escherichia coli* O157:H7 persisted in fallow soil and silt loam soil for 25-40 days (Gagliardi and Karns 2002). In our study conducted in a growth chamber, we were able to detect *E. coli* O157:H7 in soil by MPN after 28 days.

Competition between epiphytes and enteric pathogens has been studied on plants and seeds (Liao and Fett 2001; Matos and Garland 2005). Liao and Fett (2001) were able to isolate four strains of native plant microflora that inhibited *E. coli, Salmonella* and *Listeria monocytogenes* on agar medium. Survival of enteric pathogens is also dependent to some extent on the presence of indigenous nonpathogenic bacteria. Other workers have shown enhanced survival of bacterial cells introduced to the leaf surface when they are deposited on or in previously formed aggregates of other bacteria (Monier and Lindow 2005). Previous work has shown the ability of enteric bacteria to form aggregates on foliar surfaces with epiphytic bacteria (Brandl and Mandrell 2002). The role of these bacterial aggregates on the survival of *E. coli* O157:H7 in our study is unclear. The inability of *E. coli* O157:H7 to persist on spinach leaves for 14 days may be attributed to the presence of competing nonpathogenic *E. coli* bacteria, lack of formation of bacterial aggregates on spinach leaves or the presence of other epiphytic bacteria on spinach leaves as shown by the consistent populations of heterotrophic bacteria through the duration of our study.

We co-inoculated nonpathogenic *E. coli* with *E. coli* O157:H7 strains to determine their suitability for use as surrogates for future leaf and soil inoculation studies. None of these nonpathogenic *E. coli* strains encoded the *stx1*, *stx2* or *eae* genes. Additionally, these strains were negative for heat labile enterotoxin, heat stable enterotoxins a and b and cytotoxic necrotizing factors (Wachtel *et al.* 2002b) The strains were isolated from produce and therefore they were suitable candidates as surrogate micro-organisms. While recovery of these strains from plant shoots varied, strain MW 423 was not detected 21 days after inoculation. *E. coli* strains PM 3954 and MW 425 persisted for 21 and 28 days, respectively, and were recovered at greater populations than were other strains, indicating differential fitness among strains. Previous studies have documented persistence of these nonpathogenic strains in soil. Nonpathogenic *E. coli* strains, MW 416, MW 423 and MW 425, were detected in soil and within cabbage root rhizosphere for at least 7 days after cabbage was accidentally irrigated with partially treated sewage wastewater (Wachtel *et al.* 2002b). In our study, the difficulty in recovering nonpathogenic *E. coli* and *E. coli* O157:H7 from soil by direct plating emphasizes the use of selective enrichment techniques in combination with MPN methods to quantify *E. coli* populations in soil. *Escherichia coli* strains in our study did not contain antibiotic resistance, fluorescent or luminescent markers, which would have facilitated their recovery by direct plating. The use of these markers in nonpathogenic *E. coli* would have diminished the utility of these strains as surrogates for *E. coli* O157:H7 in field studies.

Overall, HPC on the plant shoots remained relatively constant. While growing plants provides surfaces for bacterial attachment, the phyllosphere is comparatively dry when not immediately subjected to spray irrigation, and it is a relatively nutrient-poor environment (Delaquis *et al.* 2007). Total aerobic populations of fresh produce at field level varied from 4·5 to 6·2 log CFU g^{-1} (Johnston *et al.* 2005). However, aerobic counts of lettuce and sprouts obtained at retail markets were 8·6 log CFU g^{-1} (Thunberg *et al.* 2002). In their study, an increase in aerobic plate counts had no relation with the presence of pathogens. There was no correlation between survival of HPC and *E. coli* O157:H7 populations by treatment. Likewise, HPC in soil were similar on days 0 and 28. Increase in soil HPC at day 7 may be attributable to resuscitation of the HPC by plant irrigation before microbiological analysis.

Results from our work show that *E. coli* O157:H7 survived for 7–14 days on young spinach leaves exposed to a single contamination event in growth chambers, when co-inoculated with nonpathogenic *E. coli.* Two *E. coli* strains MW 416 and MW 423 were not detected on spinach leaves on day 21, closely resembling the survival profile for the co-inoculated *E. coli* O157:H7 strains. Persistence of *E. coli* strains MW 416 and MW 423 on spinach leaves for 14 days in comparison with the *E. coli* O157:H7 persistence of 7 days will provide safeguard if these strains are used as surrogates for *E. coli* O157:H7 in field studies on spinach. The identification of these *E. coli* surrogates provides alternatives for using pathogenic *E. coli* O157:H7 in inoculation studies, and more work is warranted to determine their survival on other leafy green commodities.

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